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IMMUNOBIOLOGICAL BARRIERS TO XENOTRANSPLANTATION

David K.C. Cooper¹, Burcin Ekser², and A. Joseph Tector²

¹Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA, USA

²Transplant Division, Department of Surgery, Indiana University School of Medicine, Indianapolis, IN, USA

Abstract

Binding of natural anti-pig antibodies in humans and nonhuman primates to carbohydrate antigens expressed on the transplanted pig organ, the most important of which is galactose- α 1,3-galactose (Gal), activate the complement cascade, which results in destruction of the graft within minutes or hours, known as hyperacute rejection. Even if antibody is removed from the recipient's blood by plasmapheresis, recovery of antibody is associated with acute humoral xenograft rejection. If immunosuppressive therapy is inadequate, the development of high levels of T cell-dependent elicited anti-pig IgG similarly results in graft destruction, though classical acute cellular rejection is rarely seen. Vascular endothelial activation by low levels of anti-nonGal antibody, coupled with dysregulation of the coagulation-anticoagulation systems between pigs and primates, leads to a thrombotic microangiopathy in the graft that may be associated with a consumptive coagulopathy in the recipient. The most successful approach to overcoming these barriers is by genetically-engineering the pig to provide it with resistance to the human humoral and cellular immune responses and to correct the coagulation discrepancies between the two species. Organs and cells from pigs that (i) do not express the important Gal antigen, (ii) express a human complement-regulatory protein, and (iii) express a human coagulation-regulatory protein, when combined with an effective immunosuppressive regimen, have been associated with prolonged pig graft survival in nonhuman primates.

Address for correspondence:-David K.C. Cooper MD, PhD, Thomas E. Starzl Transplantation Institute, Starzl Biomedical Science Tower, W1543, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA 15261, USA, Tel:412-383-6961; Fax:412-624-1172, cooperdk@upmc.edu.

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CONFLICT OF INTEREST

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DKCC, BE, AJT – all participated in review of the literature, writing of the manuscript, and final approval of the manuscript.

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Organ xenotransplantation

When interest developed in using the pig as a potential organ source for humans, little was known about the immunology of pig-to-primate organ transplantation. Studies in the clinically-relevant (wild-type) pig-to-baboon model indicated that very rapid graft destruction occurred, usually within minutes [1–3].

Hyperacute rejection

When a pig organ is transplanted into a human or nonhuman primate, there is an immediate immune response, known as hyperacute rejection (Figure 1A). This has been defined as destruction of the graft within 24 hours, but it frequently occurs within the first hour, and is related to binding of primate natural (preformed) anti-pig antibodies to the graft vascular endothelial cells. Antibody deposition initiates complement-mediated injury of the endothelial lining, resulting in thrombosis, interstitial hemorrhage and edema that disrupts graft function [4,5].

It was subsequently determined that the most important antibodies (IgM and IgG) bind to a carbohydrate epitope, galactose- α 1,3-galactose (Gal), expressed on the pig vascular endothelium (reviewed in [6]). This oligosaccharide is present in all other mammals, with the exception of humans and Old World nonhuman primates (e.g., great apes, baboons, Old World monkeys) (reviewed by [7]). These primate species lost expression of Gal several million years ago, probably from a genetic mutation, and the absence of Gal resulted in primates making antibodies against this now ‘foreign’ antigen.

These antibodies develop during neonatal life [8,9], and are almost certainly a response to Gal-expressing viruses and microorganisms that colonize the primate’s gastrointestinal tract [10]. These ‘natural’ or ‘preformed’ antibodies differ from ‘elicited’ antibodies that develop after direct exposure to an antigen, e.g., antibodies that develop after an organ transplant.

As the causative factors associated with hyperacute rejection of a xenograft were seen to be similar to those of ABO-incompatible allograft rejection [11], a similar approach was taken to prevent rejection by depleting the recipient of these anti-pig antibodies by plasmapheresis [3] or, later, by depleting specifically anti-Gal antibodies by immunoaffinity columns [12]. In addition, again based on experience with ABO-incompatibility studies, the intravenous infusion of natural or synthetic Gal oligosaccharides was tested, which were bound by anti-Gal antibody and then excreted [13,14]. Even when combined with conventional immunosuppressive therapy, these approaches were only partially successful; they delayed antibody-mediated rejection, but the graft was lost when antibody levels recovered.

An alternative or additional approach was to administer an agent that depleted or inhibited complement, e.g., cobra venom factor, which extended graft survival significantly [15,16], but again had only a temporary effect.

When genetic modification of the organ-source pig became possible, a different approach to overcoming hyperacute rejection was suggested by Dalmaso (in the USA) [17] and, independently, by White (in the UK) [18] and their respective colleagues. The cells of humans are to some extent protected from complement-mediated injury by the presence of complement-regulatory proteins on their surfaces, e.g., decay accelerating factor (DAF, CD55), or membrane cofactor protein (MCP, CD46). Although pig cells have equivalent complement-regulatory proteins, these are less able to provide protection from the effects of human complement. Dalmaso and White suggested introducing into the pig a transgene for a human complement-regulatory protein. In the mid-1990s, this was achieved by several groups, and represent the first genetically-engineered pigs directed towards xenotransplantation (reviewed by [19]).

When the importance of Gal had been established, it was suggested that the gene that produced the enzyme that attached Gal terminally on oligosaccharide chains, α 1,3-galactosyltransferase, should be deleted or knocked-out [20]. The first α 1,3-galactosyltransferase gene-knockout (GTKO) pig was not produced until 2003 [21,22]. Initial studies showed protection from hyperacute rejection [23,24].

Acute humoral xenograft rejection

Even when hyperacute rejection was prevented, a similar form of rejection occurred, generally within a few days or weeks - acute humoral xenograft rejection (AHXR). It is also related to the deposition of antibody and complement, which activate the endothelium [25], and the effect of graft infiltration by innate immune cells (e.g., polymorphonuclear leukocytes, macrophages, NK cells) that together destroy the graft. When a GTKO pig organ is transplanted, the antibodies involved are natural antibodies directed against nonGal antigens, the exact nature of which remains uncertain, although two have been identified (see below).

The combination of GTKO and a human complement-regulatory protein was even more successful in preventing early graft failure of a transplanted pig organ [26,27].

The adaptive immune response

If both hyperacute and AHXR are prevented, but immunosuppressive therapy is inadequate, a T-cell dependent elicited antibody response develops, resulting in high levels of anti-pig IgG [28]. Binding of these antibodies to the vascular endothelium initiates histopathological changes indistinguishable from AHXR. Surprisingly, acute cellular rejection, as seen in the majority of allotransplants, has virtually never been recorded after pig-to-nonhuman primate organ xenotransplantation. This is most likely because the humoral response is more rapid and overwhelms the cellular response, though T and B cells may well be seen in the graft.

Although high doses of conventional immunosuppressive therapy, e.g., tacrolimus, mycophenolate mofetil, and corticosteroids, have delayed graft failure [29,30], they have been associated with a relatively high incidence of infectious complications. A potentially more successful approach has been with the administration of agents that are directed to

costimulation blockade, first introduced into xenotransplantation by Buhler in 2000 ([28]; reviewed in [31]).

Here again, genetic engineering of the organ-source pigs may prove beneficial (reviewed in [31]) Pigs transgenic for the T cell costimulation blockade agent, CTLA4-Ig, have been produced successfully [32], but, because of the high levels of soluble CTLA4-Ig produced, proved to immunocompromised, prohibiting their long-term survival. Subsequent efforts have been directed towards expressing CTLA4-Ig in only selected tissues, e.g., the vascular endothelium or the islets.

A second approach has been to express a mutant human Major Histocompatibility Complex (MHC) class II transactivator transgene in the pig. This mutation results in downregulation of swine leukocyte class II expression, and inhibits upregulation of expression after activation of the pig endothelial cells, thus reducing the human T cell response [33]. Recently, MHC class I-knockout pigs have been produced [34], and their effect as donors of xenografts is being investigated.

Coagulation dysfunction

Even when hyperacute rejection, AHXR, and the T cell response were successfully controlled, it became increasingly clear that abnormal coagulation within the vessels of the graft played a significant role in graft failure (reviewed by [35,36]). Coagulation dysregulation can result in the development of a thrombotic microangiopathy, in which the vasculature of the organ is steadily occluded by thrombus, resulting in ischemic necrosis of the tissues (Figure 1B) [37,38].

Small vessels in the graft become occluded by fibrin and platelet aggregation. When advanced, the loss of platelets and clotting factors in the graft may result in a consumptive coagulopathy in the recipient primate [39]. The initiating cause of the thrombotic microangiopathy almost certainly results from activation of the vascular endothelial cells by antibody, complement, and/or innate immune cells, changing the phenotype of the cells from an anticoagulant state to a procoagulant state. However, there are several known incompatibilities in the coagulation/anticoagulation factors between pig and primate that almost certainly contribute to coagulation dysregulation between graft and host [35,36]. Consumptive coagulopathy appears to develop much more rapidly in nonhuman primates with pig kidney grafts whereas a thrombotic microangiopathy predominates after pig heart transplantation, followed terminally by a consumptive coagulopathy [23,39,40].

Efforts were made to control this problem by the administration of anticoagulants and/or anti-thrombotic agents to the recipient nonhuman primate, with partial success [37], but evidence was presented indicating that increased immunosuppression was more effective than anticoagulant agents [41]. This problem is also steadily being overcome by further genetic manipulation of the organ source pig, e.g., by the insertion of an 'anticoagulant' or 'anti-thrombotic' gene, such as thrombomodulin, endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), or CD39 [35,36], resulting in extended graft survival [42].

Inflammatory response

Ezzelarab has presented evidence that an inflammatory response precedes the development of coagulation dysfunction, emphasizing the inter-relationships between the immune, coagulation and inflammatory responses [43]. In particular, the potential role of interleukin-6 (IL-6) has been highlighted [43,44], (and the beneficial effect of an IL-6R antagonist is being explored [44]).

Graft vasculopathy (chronic rejection)

Chronic rejection (graft vasculopathy) has been documented in some pig cardiac xenografts that have functioned for >3 months in baboons, and has a similar histopathological appearance to that seen after allotransplantation (Figure 1C) [45]. Some encouragement, however, is provided by the ongoing studies of Mohiuddin et al in which graft vasculopathy has not been reported in heart grafts from GTKO/hCD46 pigs expressing human thrombomodulin, with follow-up in one case for more than two years [42].

Remaining barriers

The immunobiological problems associated with transplantation of pig livers [46] and lungs [47] are more complex, and to date have limited graft survival to days rather than weeks, months or years (reviewed in [48]). Humans are known to have natural antibodies to at least two other antigens on pig cells, namely N-glycolylneuraminic acid (NeuGc) (reviewed in [49]) and β 1,4 N-acetylgalactosaminyltransferase [50]. As most nonhuman mammals express NeuGc, including Old World nonhuman primates (even chimpanzees), it is difficult to investigate its effect in *in vivo* experimental models, e.g., pig-to-baboon organ transplantation. Most information to date, therefore, has been obtained from staining of pig tissues for expression of NeuGc, and laboratory assays. There is increasing evidence that this antibody-antigen interaction will need to be prevented if a pig organ or cells are transplanted into a human patient. The recent production of pigs in which the gene responsible for producing NeuGc has been deleted (as well as that for Gal) is a major step towards this goal [51].

Cell and tissue xenotransplantation

Pigs could also act as a source of other tissues, such as pancreatic islets (for the treatment of diabetes), neuronal cells (for conditions such as Parkinson's disease), corneas (for patients with corneal blindness), and red blood cells (for clinical transfusion). The immunobiology of graft injury is similar to that seen in pig organs, but a T cell-induced response appears to play a more important role, as in allografts.

Considerable attention has been paid to the transplantation of porcine islets of Langerhans as a means of providing a source of insulin in patients with Type 1 diabetes (reviewed in [52]). Techniques are available, e.g., by the use of an insulin-specific promoter, that enable a desired transgene to be expressed in the islets alone, thus negating any potential detrimental effects (e.g., systemic anticoagulation) that might result from widespread expression in the pig [53]. However, success has not yet been consistent, and further study is required.

The cellular immune response can be inhibited by currently-available immunosuppressive agents, particularly by those that result in blockade of T cell costimulation. Both wild-type and genetically-engineered *adult* porcine islets have been demonstrated to maintain normoglycemia in diabetic monkeys for periods in excess of 1 year [53–55]. Wild-type *neonatal* pig islets have also maintained normoglycemia in diabetic monkeys for >6 months [56,57].

Induction of tolerance to pig organs and cells

The ideal goal in both allotransplantation and xenotransplantation is the induction of immunological ‘tolerance’, in which the immune system of the recipient is manipulated so that the transplanted organ or cells are accepted as ‘self’ with no effort made to reject them. The identification and availability of the ‘donor’ prior to the transplant allows the procedure to be ‘timed’, and may facilitate the induction of tolerance to the transplanted organs of cells. Efforts in this respect have been made through inducing hematopoietic cell chimerism [58,59] or by pig thymic transplantation [24], but without complete success to date.

Conclusion

With the increasing variety of genetically-engineered pigs (Table 1) and immunosuppressive and anti-inflammatory agents now becoming available, it is likely the remaining immunobiological problems will be resolved.

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ABBREVIATIONS

Gal	galactose- α 1,3-galactose
GTKO	α 1,3-galactosyltransferase gene-knockout
NeuGc	N-glycolylneuraminic acid
NeuGcKO	N-glycolylneuraminic acid gene-knockout

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HIGHLIGHTS

- Several barriers need overcoming if pig organs are to be transplanted successfully
- Both the innate and adaptive immune responses need to be overcome
- Additional barriers include coagulation dysfunction and an inflammatory response. Most barriers can be overcome by the genetic engineering of the pigs

Hyperacute Rejection (HAR) (Figure 1A)

Complement-mediated injury associated with the immediate binding of preformed anti-pig antibodies to antigens expressed on the vascular endothelium of the pig organ, e.g., Gal α 1,3Gal. Defined as occurring within the first 24 hours.

Acute Humoral Xenograft Rejection (AHXR)

A delayed antibody-mediated response often, but not always, associated with the production of elicited antibodies, that leads to vascular endothelial activation and injury caused by complement and cellular components of the immune system, e.g., NK cells and macrophages. Histopathology similar to that of HAR. May take days or weeks to develop and cause graft failure.

Acute Cellular Rejection (ACR)

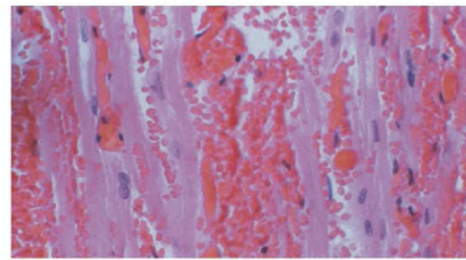
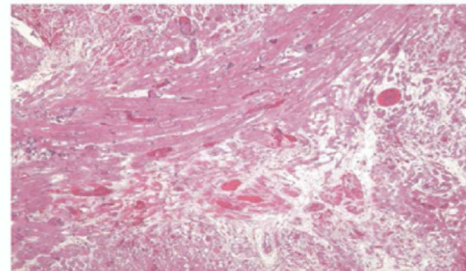
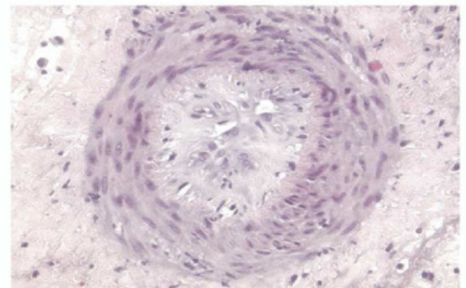
A cellular response, including T cells, that is similar, but not identical, to that seen after allotransplantation. Perhaps surprisingly, it has rarely been described after pig-to-primate organ xenotransplantation, and has not proved a common problem. This is possibly because T cell activation leads to a more rapid antibody response that results in AHXR before significant T cell infiltration occurs in the graft.

Thrombotic Microangiopathy (TM) (Figure 1B)

Fibrin deposition and platelet aggregation result in thrombosis within the vessels of the graft, even in the absence of an elicited antibody response. Endothelial cell activation, e.g., by antibodies, may result in a change from the normal anticoagulant state of the vascular endothelium to a procoagulant state, resulting in fibrin and platelet deposition. This is probably associated with the numerous molecular incompatibilities between the pig and primate coagulation systems that prevent maintenance of an anticoagulant state after xenotransplantation.

Chronic Rejection (Figure 1C)

Has been documented in pig cardiac grafts that survive for >3months in baboons. Similar histopathological appearance to that seen after allotransplantation. Pathogenesis is poorly understood.

**Figure 1A: Hyperacute Rejection****Figure 1B: Thrombotic Microangiopathy****Figure 1C: Chronic Rejection****Figure 1.**

Summary of the known major immunologic barriers to pig-to-primate heart transplantation. (Reproduced with permission from Zhu X, et al, *J Heart Lung Transplant* 2007;26:210–218.)

Table 1

Genetically-engineered pigs produced for xenotransplantation research

Complement regulation by human complement-regulatory gene expression:	
CD46 (membrane cofactor protein)	
CD55 (decay-accelerating factor)	
CD59 (protectin or membrane inhibitor of reactive lysis)	
Gal or nonGal antigen ‘masking’ or deletion:	
human H-transferase gene expression (expression of blood type O antigen)	
endo-beta-galactosidase C (reduction of Gal antigen expression)	
α 1,3-galactosyltransferase gene-knockout (GTKO)	
Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene-knockout (NeuGc-KO)	
β 4GalNT2 (β 1,4 N-acetylgalactosaminyltransferase) gene-knockout (β 4GalNT2-KO)	
Suppression of cellular immune response by gene expression or downregulation	
CIITA-DN (MHC class II transactivator knockdown, resulting in swine leukocyte antigen class II knockdown)	
Class I MHC-knockout (MHC-I-KO)	
HLA-E/human β 2-microglobulin (inhibits human natural killer cell cytotoxicity)	
human FAS ligand (CD95L)	
human GnT-III (N-acetylglucosaminyltransferase III) gene	
porcine CTLA4-Ig (Cytotoxic T-Lymphocyte Antigen 4 or CD152)	
human TRAIL (tumor necrosis factor-alpha-related apoptosis-inducing ligand)	
Anticoagulation and anti-inflammatory gene expression or deletion	
von Willebrand factor (vWF)-deficient (natural mutant)	
human tissue factor pathway inhibitor (TFPI)	
human thrombomodulin	
human endothelial protein C receptor (EPCR)	
human CD39 (ectonucleoside triphosphate diphosphohydrolase-1)	
Anticoagulation, anti-inflammatory, and anti-apoptotic gene expression	
human A20 (tumor necrosis factor-alpha-induced protein 3)	
human heme oxygenase-1 (HO-1)	
Porcine asialoglycoprotein receptor 1 gene-knockout (ASGR1-KO) (decreases platelet phagocytosis)	
Human signal regulatory protein α (SIRP α) (decreases platelet phagocytosis by ‘self’ recognition)	
Prevention of porcine endogenous retrovirus (PERV) activation	
PERV siRNA	